

Isolation and Molecular Identification of Two Fungal Strains Capable of Degrading Hydrocarbon Contaminants on Saudi Arabian Environment

Amr A. El Hanafy, Yasir Anwar, Saleh A. Mohamed, Saleh Mohamed Saleh Al-Garni, Jamal S. M. Sabir, Osama A. H. Abu Zinadah, Mohamed Morsi Ahmed

Abstract—In the vicinity of red sea about 15 fungi species were isolated from oil contaminated sites. On the basis of aptitude to degrade the crude oil and DCPIP assay, two fungal isolates were selected amongst 15 oil degrading strains. Analysis of ITS-1, ITS-2 and amplicon pyrosequencing studies of fungal diversity revealed that these strains belong to *Penicillium* and *Aspergillus* species. Two strains that proved to be the most efficient in degrading crude oil was *Aspergillus niger* (54%) and *Penicillium commune* (48%) Subsequent to two weeks of cultivation in BHS medium the degradation rate were recorded by using spectrophotometer and GC-MS. Hence, it is cleared that these fungal strains has capability of degradation and can be utilize for cleaning the Saudi Arabian environment.

Keywords—Fungal strains, hydrocarbon contaminants, molecular identification, biodegradation, GC-MS.

I. INTRODUCTION

DURING past decades petroleum and its product play a big role in polluting the environment. Peoples are using a lot of automobiles and machinery vehicles, which is directly related with the use of motor oil. After using motor oil (Jet or diesel fuels) are spill, which contaminate our natural environment with hydrocarbons such as PAHs. These polyaromatic hydrocarbons spread on the ground water surface and partition into ground water. During raining season these PAHs goes towards rivers, then to sea and enter to food chain of human beings. PAHs represent hazardous compounds which can cause serious diseases such as cancer, Asthma and hormonal diseases [1]-[4].

From the last two decades fungi have drawn attention as it can play important role in bioremediation. For long time researchers were mainly focused on the use of bacteria., but recently fungi receive considerable attention for its potential towards bioremediation as it produce enzymes which are not only used in lignin breakdown but also can degrade a wide range of recalcitrant pollutants such as Polyaromatic

Amr A. El Hanafy is with the Department of Biological Sciences, Faculty of Science, PO Box 80203, King AbdulAziz University, Jeddah, 21589, KSA, Nucleic Acids Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technology Applications, Borg El-Arab, PO Box 21934, Alexandria, Egypt (e-mail: amrr220@yahoo.com).

Saleh A Mohamed is with Biochemistry department, Faculty of science, King Abdulaziz University, Jeddah, 21589, Saudi Arabia.

Saleh Al-Garni, Jamal SM Sabir, Osama AH Abu Zinadah, and Mohamed Morsi Ahmed were with Department of Biological Sciences, Faculty of Science, PO Box 80203, King AbdulAziz University, Jeddah, 21589, KSA.

hydrocarbons, chlorophenols, and pesticides [5]. Fungi produce hyphae that penetrates the soil and has an advantage our bacteria to reach the surface in order to clean PAHs from contaminated soil [6], [7].

There are few studies which have been carried out for screening, isolation and characterization of hydrocarbons degrading fungi, which shows it importance in the application of bioremediation [3], [8]-[12]. Since researcher are focusing on the bioremediation of polyaromatic and polycyclic but it would be interesting to be able to find the tropical fungi that is capable to degrade long chain hydrocarbon. The aim of our study was to isolate fungi that have the ability to degrade almost 100% of crude oil and can be used as environment (Soil and water) cleaner in near future.

II. MATERIALS AND METHODS

A. Sampling

For the isolation of oil degrading Fungi six samples were collected from Red sea shore Saudi Arabia. All of the six collection points were located near Yanbu industrial Area (24.0833° N: 38.0000° E).

B. Isolation and Identification

Czapak Dox Agar (CDA) and Potato dextrose Agar (PDA) were used for the isolation of fungi. Then pure isolates were tested for the ability to grow on Bushnell haas media, composed of: K₂HPO₄ (1 g/l), MgSO₄ (0.2 g/l), KH₂PO₄ (1 g/l), CaCl₂ (0.02 g/l), NH₄NO₃ (1 g/l) and FeCl₂ (0.05 g/l) with 1% of crude oil (ARAMCO company) at 30°C for one week [13], [14]. Fifteen different isolates were selected for further studies based on growth on BHS medium

C. Molecular Identification

Approximately 0.5 g of fungal hyphae was taken from test tube containing fungal isolate. After that, hyphae were incubated in 100 µl lyticase solution at 30°C for 60 minutes. In order to degrade protein from the crude sample, 20 µl proteinase K were added and incubated at 55°C for 90 min. Furthermore, sample was incubated for two hours at 65°C. Finally, about 10 µl of these samples were used for PCR amplification.

PCR program was set according to the conditions described by [15]. Briefly, for the 25 µl PCR reactions, the primers ITS1 (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS2 (5' TCC TCC GCT TAT TGA TAT GC 3') were used [16]. The

Master Mix for PCR contained 10 μ M deoxynucleotides, 0.5 μ M primers, 1.5 mM MgCl₂, 10 μ M dNTPs, and 1 x buffer. PCR was set 35 cycles and the amplification was carried out at as following: 94°C for 1 min; annealing at 55.5°C for 2 min and extension at 72°C for 2 min; final extension at 72° for 10 min and 4°C incubation at the end of last cycle.

D. PCR Product Sequencing and BLAST Analysis

The results from the sequencing data of the three isolates were sent to Gene Bank and after the accession numbers was given by Gene Bank molecular evolutionary analyses of these 3 strains based on ITS-1 and ITS2 was conducted using 9 different *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium commune* from Gen Bank (<http://www.ncbi.nlm.nih.gov>). The evolutionary distances were computed using the Maximum Composite Likelihood method [17] and are in the units of the number of base substitutions per site. The evolutionary analyses and phylogenetic tree was constructed using MEGA version 4 [18].

E. DCPIP Assay

DCPIP technique was used with slight modification for biodegradability of fungal isolates [19]. After one week of growth 1 cm² of fungal hyphae were picked from petri dish and transfer into 100 ml BHS medium to 500 ml conical flask containing, 0.1% (v/v) Tween 80, 1% crude oil and 0.016 mg/100 ml of redox indicator. All the flasks were incubated for two weeks at 30C. After two weeks of incubation color change from dark blue to colorless that indicates that these isolate has the ability to degrade crude oil.

F. Gravimetric Analysis

For gravimetric analysis, an equal volume of dichloromethane was added to the liquid medium containing crude oil. The extracted crude oil was analyzed according to method by [20] with slight modification.

G. GC-MS

Using Mass Spectra Gas chromatography (Shimadzu GC-Q2010, Japan)-flame ionization detector (FID) end metabolite of oil degradation by Y1 and Y4 was determined as described by [21]. After of Cultivation of isolates in Bushnell haas media at 30°C with 180 rpm for two weeks, remaining oil was extracted using separating funnel in the presence of dichloromethane. Calcium sulfate (drying agent) was used in order to remove the remaining moisture because it can affect the GC-MS column. GC-MS program was set as 60°C for 2 mins, increase 6°C until reaches 300°C for 15 mins. Operating temperature for the injector was 300°C and detector was 320°C. Nitrogen was used as carrier gas. No internal standards were employed. Total time for one GC run was less than 30 min.

III. RESULTS AND DISCUSSION

Results of this study revealed that fifteen fungal isolates were tested for insure their ability to biodegrade crude oil, two fungal strains demonstrated perfect biodegradation ability,

Aspergillus niger and *Penicillium commune* as shown Tables I and II.

A. Molecular Identification

ITS-1 and ITS-2 was amplified using specific primers. PCR product was analyzed in 3% agarose gel stained with ethidium bromide. These isolates were molecularly identified using ITS-1 and ITS-2 primers as *Aspergillus niger* (KR137638), and *Penicillium commune* (KR137639). The phylogenetic tree of these three sequences (KR137638 and KR137639) was constructed against 9 different fungi from GenData Bank (<http://www.ncbi.nlm.nih.gov>) using MEGA version 4 as shown in Fig. 1. The optimal tree with the sum of branch length = 36.19313893 is shown. The data showed that these three species laid in the same groups with many of *Aspergillus* and *Penicillium* species from GenData Bank.

B. DCPIP Assay

The screening method employed in the current study depends on color changing ability of fungal isolates inoculated in the presence of redox indicator, this helps identify the fungal isolates that have the capability to utilize crude oil as a carbon source in the presence of redox indicator. We were able to identify our isolate using this technique, as they have the potential to utilize crude oil as a sole carbon source. A similar study was also carried out by [22]. There are three main indicators that help identify fungal isolates with the ability to degrade crude oil, change in color of the media used for culturing from blue to colorless and reduction in the quantity of crude oil and fungal proliferation. The mechanism utilized by fungi to biodegrade crude oil occurs by incorporation of an electron acceptor. The presence of DCPIP in the culture medium ascertains the degradation process due to the fact that the reagent changes color from blue (oxidized) to colorless (reduced) [19].

C. Spectrophotometric Analysis

Fungal species isolated were grown in the presence of 1% crude oil for 2 week with constant shaking. Latter, the microbial growth and crude oil biodegradation were assessed with spectrometer. As discussed in Table I, the strains Y1 (*Aspergillus niger*) and Y4 (*Penicillium commune*) exhibited highest level of crude-oil biodegradation, degrading 54 and 48% of the oil, respectively. Among the isolated strains, Y1 (*Aspergillus niger*) with 54% crude oil degradation proved to be the most efficient. In a similar study [23] documented the hydrocarbon degrading capabilities of bacteria were spectrophotometrically determined. All spectrophotometric analyses were carried out at 400 nm wavelength whereas the degradation kinetics were carried out at 540 nm.

In the present work *Aspergillus niger* and *Penicillium documbens* were the most efficient fungal isolates demonstrating higher biodegradable activity [24], indicated that *Aspergillus niger* have very active degradation capabilities of four kinds of oil compounds, Durb oil, Escravos light, Arabian light and Bonny light. Additionally, eight other genera from, *Aspergillus*, *Penicillium* and *Fusarium* species were also efficient metabolizers of hydrocarbons [25], [26].

Bartha and Atlas [27] in their review mentioned 14 fungal genera with the potential to degrade hydrocarbons isolated from an aquatic environment. The evolution of the

hydrocarbon mixture depends on the nature of the oil, microbial community, and environmental factors which impact microbial activities.

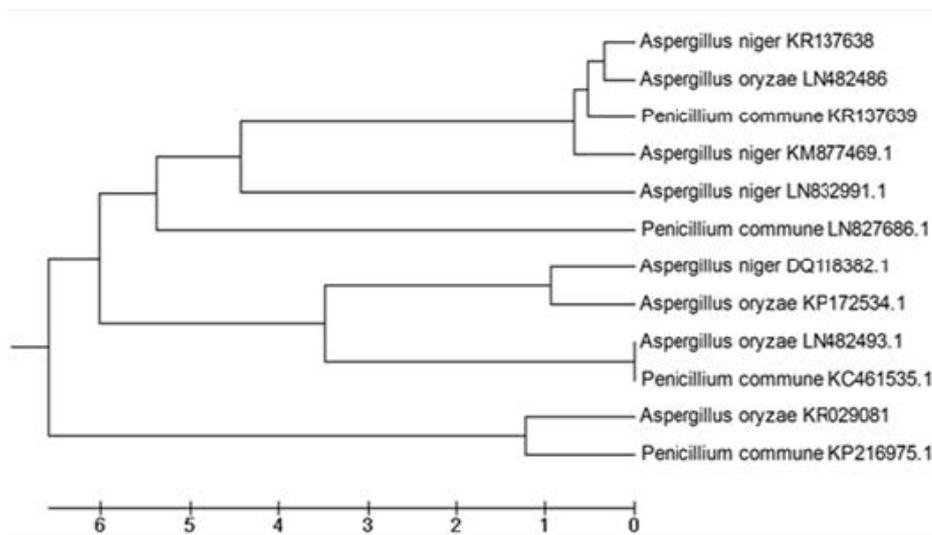


Fig. 1 Comparative phylogenetic analysis of the *Aspergillus niger* (KR137638) and *Penicillium commune* (KR137639) with different *Aspergillus* and *Penicillium* strains from GenBank

TABLE I
SPECTROPHOTOMETRY ANALYSIS OF FUNGAL ISOLATES

ID	Strain Name	Accession No.	% degradation
Y1	<i>Aspergillus niger</i>	KR137638	54
Y4	<i>Penicillium commune</i>	KR137639	48

D. GC-MS Analysis

The percentage of crude oil degradation by the isolate Y1 (54%) and Y4 (48%) was recorded respectively. Major peak

compounds at the retention time of 6.250, 6.945, 7.600, 8.225, 9.380 and 10.430 minutes were identified by comparing the data with standard library compounds as tetradecane, pentadecane, hexadecane, heptadecane, tetracosane and heptacosane. All these compounds were present in crude oil but some of them were degraded by fungal isolates as shown in Table II.

TABLE II
BIOREMEDIATION OF CRUDE OIL AFTER 2 WEEKS OF INCUBATION AT 30°C, ANALYZED BY GC-MS. DEGRADED SAMPLE USING Y1 AND Y4 AGAINST CONTROL

Compound	Retention Time (min)	Molecular weight	Peak Area (Control)	Peak Area (Y1)	Reduction Area (%)
Tetradecane	6.250	198	356526	207968	58
Pentadecane	6.945	212	986550	539161	54
Hexadecane	7.600	226	895566	425154	47
Heptadecane	8.225	240	1156283	604615	52
Tetracosane	9.380	338	970256	447257	46
Heptacosane	10.430	380	596525	299770	50

Compound	Retention Time (min)	Molecular weight	Peak Area (Control)	Peak Area (Y4)	Reduction Area (%)
Tetradecane	6.250	198	356526	195225	54
Pentadecane	6.945	212	986550	385252	39
Hexadecane	7.600	226	895566	375460	41.9
Heptadecane	8.225	240	1156283	485652	42
Tetracosane	9.380	338	970256	456585	47
Heptacosane	10.430	380	596525	235626	39

All the hydrocarbons present in crude oil that were detected using GC-MS analysis were effectively degraded by the bacterial isolated from Yanbu region. Results from spectrophotometric analysis were corroborated by GC-MS analysis. The isolates efficiently degraded hydrocarbons like tridecane, hexadecane, heptadecane, tetracosane, heptacosane, octacosane, nonacosane [28]. Similar work was done by [29], on *saccharomyces cerevisiae* that had around 49% degradation

of crude oil. It is necessary to identify and study the other groups of microorganisms that have the potential to act on long-chain hydrocarbons, such as soil actinomycetes and other fungal isolates, in the Yanbu area.

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